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Alternative Splicing of the FMR1 Gene in Human Fetal Brain Neurons

Tao Huang, Lan-Ying Li, Yan Shen, Xue-bin Qin, Zhi-Ling Pang, and Guan-Yun Wu

National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing (T.H., Y.S., X.-b.Q., G.-Y.W.); and Institute of Endocrinology, Tianjin Medical University, Tianjin (L.-Y.L., Z.-L.P.), People's Republic of China

The alternative splicing expression of the FMR1 gene was reported in several human and mouse tissues. Five regions of FMR1 gene can be alternatively spliced, but the combination of them has not been investigated fully. We reported here the analysis of alternative splicing pattern of the FMR1 gene in cultured fetal human neurons, using a RT-PCR and cloning strategy. Eleven splicing types were cloned and different isoforms were not equally represented. The dominant isoform represents nearly 40%, and the other isoforms were relatively rare. One isoform has a different carboxyl-terminus. Most of the alternative spliced regions appear hydrophilic; thus, they may locate on the surface of the FMR1 protein.

KEY WORDS: FMR1 gene, alternative splicing, neuron, development

INTRODUCTION

Alternative splicing has been found in many genes and can introduce functional diversity into the protein products of a single gene. In most cases this gives rise to protein isoforms sharing extensive identity and varying only in specific domains, thus allowing the fine regulation of gene functions. The alternative splicing expression of FMR1 gene was reported in a variety of human and mouse tissues [Verheij et al., 1993; Verkerk et al., 1993; Ashley et al., 1993a]. The down-regulation of FMR1 gene expression is the cause of the Fragile X [Fra(X)] syndrome [Verkerk et al., 1991]. The FMR1 gene encodes a cytoplasmic RNA binding protein, which can bind its own mRNA, as well as some uncharacterized mRNAs [Ashley et al., 1993b; Gibson et al., 1993; Siomi et al., 1994]. The high level expression of FMR1 gene in neurons, especially in the hippocampus, suggests that it may play a role during the development of the central nervous system (CNS), possibly in cell migration and differentiation [Devys et al., 1993; Hinds et al., 1993; Abitol et al., 1993].

Five regions of the FMR1 gene can be alternatively spliced, but the combination of them has not been investigated fully. We analyzed the subregion of the FMR1 transcripts which encompasses the sequences known to be alternatively spliced, in neurons of human fetal brain. Our results showed that there are 11 isoforms, including one that may encode a truncated protein with a different C-terminus.

MATERIALS AND METHODS

The cerebral cortex of a 10 week fetus was dissected under sterile condition. Tissues were cut in cubes of 0.5–1.0 mm^3, resuspended in Dulbeccos's modified eagle medium (DMEM), and passed through a stainless steel net (100 μm pore size) to separate the cells. The cells were cultured in DMEM/Ham's F12 (1:1) with 10% fetal calf serum, in 5% CO_2 at 37°C. After 48 hours, the cells were further maintained in serum-free medium, supplemented with 5 mg/ml of insulin, 10 mg/ml of human transferrin, 100 mM of putrescine, 30 nM of sodium selenite, 5 mg/ml of glucose, and 10 mM of HEPES. After 48 hours of serum-free culture, the proliferation and growth of glial cells were suppressed, and most cells were neurons as observed under microscope.

RNA was isolated according to Chomczynski et al. [1987]. Poly(A)RNA was separated using the QuikPrep Micro mRNA Purification Kit (Pharmacia, Piscataway, NJ) and cDNA was synthesized with First Strand cDNA Synthesis Kit (Pharmacia, Piscataway, NJ) as described by the manufacturer. Twenty picamoles of primer 27X31 were added as 3′ primers in first strand synthesis. First strand cDNA was extracted with phenol and chloroform before precipitation with ethanol, and was used as template for PCR. PCR reaction was run in 100 ml, containing 500 mM each of the four deoxynucleotide triphosphates, 50 mM KCl, 2 mM $MgCl_2$, in 10 mM Tris-HCl (pH 8.3) buffer, and 2 units of Taq polymerase. The reaction was heated to 94°C for 5 min, followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at

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Address reprint requests to Yan Shen, Department of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences, CAMS and PUMC, 5, Dong Dan San Tiao, Beijing 100005, P.R. China.

60°C), and extension (1 min and 45 sec at 72°C). Final extension was at 72°C for 7 min. The primers were K5 and 27X31. PCR products were treated with Klenow (Promega, Madison, WI), phosphorylated with T4 polynucleotide kinase (Biolabs, Beverly, MA), and then ligated to pUC18/SmaI/BAP (Pharmacia, Piscataway, NJ). Other general methods were all performed with standard methodology [Sambrook et al., 1989].

One milliliter aliquot of culture of individual colonies was used as template in PCR reaction. Amplification was carried out as described above, except that the primers were 27XM7/HFP1 or 27XM7/27X31, and the total reaction volume was 50 ml, with 1 unit of Taq polymerase. Ten milliliters of PCR product were digested with 1 unit of HinfI (Biolabs, Beverly, MA) or HincII (Biolabs, Beverly, MA) for 2 hours at 37°C before being analyzed by 6% PAGE. $MgCl_2$ concentration was adjusted for optimal endonuclease activity. The primers used are

K5 (5′-GATGCAGTGAAAAAAGCTAGAAGC-3′)

27XM7 (5′′-CAGCACCCATTTTTCTCAACCTAACAGTAC-3′′)

HFP1 (5′-GCGATGCTGTCCTTTGTTCCCA-3′)

27X31 (5′-CAAATCTGAAAATTGTTGTTGCTTATGGCC-AATACC-3′)

RESULTS AND DISCUSSION

The 1.3 kb region, which covers about 50% of the total FMR1 coding sequence and all five sequences previously known to be alternatively spliced, was amplified from poly(A)RNA of human fetal brain neurons (Fig. 1A). To examine exon use, the PCR product was subcloned en mass into a plasmid vector. Forty cDNA clones were analyzed by nested PCR combined with endonuclease digestion to determine which combination of alternative regions was present in each clone. The presence of regions C2 and D was identified by the presence of a unique restriction site (HinfI and HincII, respectively) within the alternative spliced region. In the cases of alternative splicing region B, C1, and E, the nested PCR generated smaller fragments (Fig. 2).

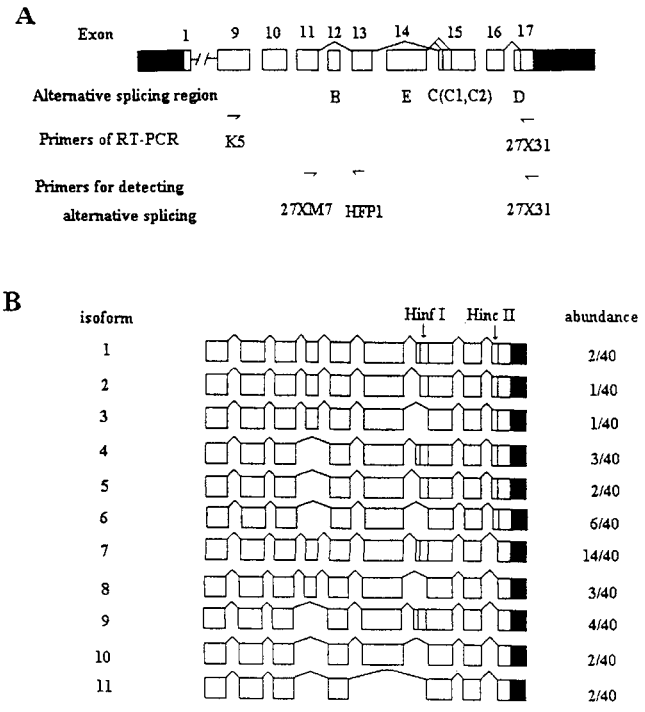

Fig. 1. **A:** Part of the FMR1 gene encompassing the alternative splicing regions. The primers used in analysis are presented beneath. **B:** The eleven splicing types detected in the neurons of human fetal brain. The black boxes represent non-coding regions. Hinf I and Hinc II are restriction endonucleases used in analyzing the existence of C and D regions, respectively.

Eleven isoforms were found in fetal neurons (Fig. 1B). The splicing sites were confirmed by sequencing (data not shown). Preliminary analysis of the splicing pattern revealed that different isoforms were not present equally. The dominant isoform was isoform 7 with just region D [5′ part of exon 17, see Verkerk et al., 1993] spliced out, represented a fraction of nearly 40%. The other isoforms were relatively rare being present less than 10% each. Only one isoform with the E region (exon 14) spliced out was found, in 2 of 40 clones. Exon 12 (region B) was absent in about 50% (19 of 40) clones. The most 5' acceptor site of exon 15 was used in 23 of 40 clones, while the second and third sites were used in 3 and 14 clones, respectively. The second acceptor site in exon 17 was used more often than the first (25 versus 15 clones).

The hydrophobicity and secondary structure predictions for the different isoforms were studied. Of the 40 clones analyzed, two (isoform 11) were with B, C1, C2, D, and E spliced out, resulting in a different carboxyl terminus with a hydrophobic region instead of an interspersion of hydrophilic and hydrophobic sequences (not shown). The b-turn rich C-terminal domain present in the other isoforms, which includes a RGG box, conserved in many RNA-binding proteins, was truncated by this frame shift. Structure prediction also showed that most of the other alternatively spliced regions were hydrophilic. These regions may thus be located on the surface of FMR1 protein and might modulate RNA/protein or protein/protein interactions.

Our results confirm the extensive diversity of alternative transcripts of the FMR1 gene. Verkerk et al. analyzed the alternative splicing of B, C1, C2, and D regions. They detected six isoforms in various mouse and human tissues, and reported the unequal abundance of each isoform [Verkerk et al., 1993]. Ashley et al. found 12 isoforms by analyzing the B, C1, C2, and E regions in human and mouse brain. Six of the isoforms included splicing out of the E region, thus resulting in novel carboxyl termini [Ashley et al., 1993a]. But these studies only covered parts of the alternative splicing regions of FMR1 gene. The combination of these regions had not yet been investigated. We used a "cloning" strategy similar to that of Thackeray et al.: the total amplified transcripts were cloned and a large sample of clones was subjected to intensive analysis [Thackeray et al., 1994]. This allowed us to obtain a more accurate representation of splicing types and patterns. Thackeray et al. have demonstrated that the number of colonies of each splicing isotype does reflect the abundance of each type of transcript [Thackeray et al., 1994].

Up to date, the real function of FMR1 gene is still unclear. The multiple phenotypes resulting from the ab-

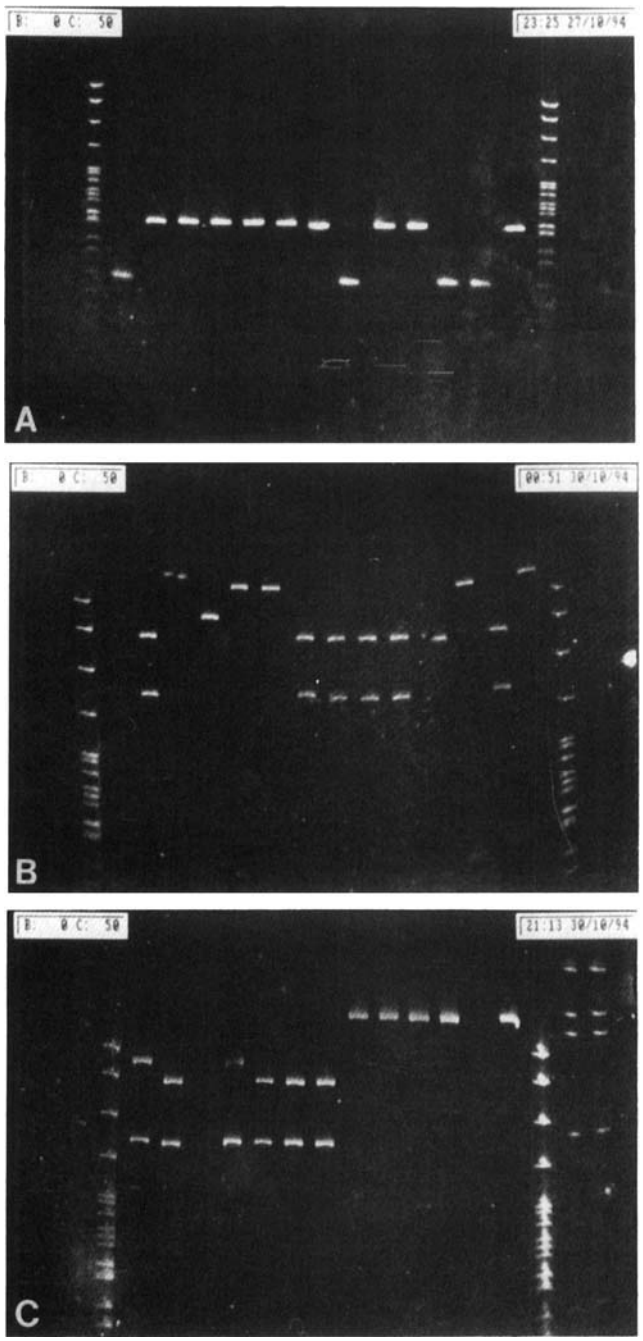

Fig. 2. Electrophoresis of the PCR products or restriction enzyme digested PCR products. **A:** The existence of B region was determined by the length of PCR products, either 150 bp (B+) or 87 bp (B−), as amplified by primers 27XM7 and HFP1. **B:** Identification of the existence of C2 region. Digested with restriction enzyme Hinf I, the PCR products amplified by primers 27XM7 and 27X31 could show one band (C2−) or two bands (C2+). **C:** Identification of the existence of D region. Digested with restriction enzyme Hinc II, the PCR products amplified by primers 27XM7 and 27X31 could show one band (D−) or two bands (D+). The existence of C1 and E regions were determined by the length of PCR products in B or/and C. M: pBR322/Msp I, M′: pBR322/BstN I.

normal expression of FMR1 gene may be caused by the abnormal expression of the downstream genes regulated by FMR1, as suggested by the RNA-binding character of FMR1 protein and the protein/protein interactions between FMR1, FXR1, and FXR2 [Siomi et al., 1995; Zhang et al., 1995]. Alternative splicing of the FMR1 gene may contribute to functional diversity. It may generate up to 24 different mature transcripts, corresponding to a maximum of 20 different protein isoforms, that may have different RNA recognition specificity or protein binding abilities.

Different isoforms of FMR1 expression products coexist in the same tissues, as revealed by RT-PCR and Western blot. The alternative splicing dose not appear tissue-specific, but the relative abundance of different transcripts seems to be different in different tissues [Verheij et al., 1993; Verkerk et al., 1993; Ashley et al., 1993a]. Investigation of splicing pattern during development of CNS, in different regions of brain, would be interesting. Further work is needed concerning the identification of the corresponding protein isoforms, and their spatial and temporal distribution. Western blot revealed at least four protein isoforms, but isoform-specific antibodies have to be raised to distinguish between these isoforms. Our results confirm that isoform 1 (the longest) is a very minor one, as suggested by Sittler et al. [1996].

The disturbance of the development of neuron by abnormal expression of the FMR1 gene may lead to the mental retardation, and it will be important to study the function of FMR1 in the development of CNS. Analysis of its developmental splicing pattern may constitute a first step.

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